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Inactivation of the E-Cadherin Gene in Primary Gastric Carcinomas and Gastric Carcinoma Cell Lines

Gen Tamura,[1,3] Ken Sakata,[1] Satoshi Nishizuka,[1] Chihaya Maesawa,[1] Yasushi Suzuki,[1] Takeshi Iwaya,[1] Masanori Terashima,[2] Kazuyoshi Saito[2] and Ryoichi Satodate[1]

Departments of [1]Pathology and [2]Surgery, Iwate Medical University School of Medicine, 19-1 Uchimaru, Morioka 020

We investigated the E (epithelial)-cadherin gene for mutations and loss of heterozygosity (LOH) in 24 primary gastric carcinomas (12 differentiated and 12 undifferentiated types, including 3 signet-ring cell carcinomas), as well as 4 gastric carcinoma cell lines of the undifferentiated type (MKN-45, GCIY, HGC-27 and GT3TKB). We utilized PCR-SSCP and RT-PCR followed by direct sequencing to detect gene mutations and skipped exons, and RT-PCR-SSCP to examine LOH. In primary carcinomas, gene mutations or skipped exons were detected in 4 of 9 (44%) undifferentiated carcinomas of the scattered type, including 2 signet-ring cell carcinomas, and in none of the 3 undifferentiated carcinomas of the adherent type and 12 differentiated carcinomas. Demonstrated mutations of the E-cadherin gene included an 18 bp deletion (codon 418–423) and a 3 bp deletion (codon 400, calcium-binding domain), both located in exon 9. Skipping of exon 9 with a 1 bp insertion at codon 337, and skipping of exon 8 with a 1 bp deletion at codon 336, also were detected. LOH was confirmed in all of the carcinomas in which gene mutations or skipped exons (3/3 informative cases) were demonstrated. The MKN-45 cell line exhibited an 18 bp deletion at the exon 6-intron 6 boundary with loss of the wild-type allele, and 2 of the remaining 3 cell lines (HGC-27 and GT3TKB) had lost expression without detectable structural alteration of the E-cadherin gene. These data provide support for classic two-hit inactivation of the E-cadherin gene in a high percentage of undifferentiated carcinomas of the scattered type.

Key words: E-cadherin — Gastric carcinoma — Gene mutation — Loss of heterozygosity

Gastric carcinomas can be classified as either differentiated carcinomas forming tubular or papillary structures or undifferentiated carcinomas in which such structures are inconspicuous.[1] Recent genetic studies have revealed several genetic alterations, such as activation of oncogenes and inactivation of tumor suppressor genes, in a variety of human malignancies. In gastric carcinomas, it has been suggested that the differentiated and undifferentiated types of carcinoma may develop through different genetic pathways.[2] However, genetic alterations which are highly prevalent and thus have been considered to play important roles in gastric carcinogenesis, such as *p53* gene mutations[3] and deletions of 5q[4,5] and 18q,[6] are present in both tumor types. Therefore, these two types of carcinoma may develop through several common genetic alterations and thereafter progress toward different morphologies by accumulation of additional genetic events.

The most characteristic feature which distinguishes differentiated from undifferentiated carcinomas of the stomach is the tubular or papillary structures described avove. In addition to this feature, undifferentiated carcinomas often lack tight intercellular adhesion between carcinoma cells and can consequently be further divided into two groups; scattered and adherent types.[1] This reduced adhesiveness in undifferentiated carcinomas of the scattered type is thought to contribute to the invasive capacity of carcinoma cells.[7,8]

E (epithelial)-cadherin is a member of a family of transmembrane glycoproteins that are responsible for calcium-dependent cell-to-cell adhesion and also appear to play a role in organogenesis and morphogenesis.[9] Loss or reduction of E-cadherin expression has been demonstrated immunohistochemically in several types of human carcinomas, including gastric carcinomas.[1,10–13] However, immunohistochemical assessment of E-cadherin expression in gastric carcinoma remains controversial. Shimoyama and Hirohashi[1] have reported that E-cadherin expression was absent in 4 of 28 (14%) undifferentiated carcinomas of the scattered type, while Mayer *et al.*[10] found no immunoreactivity for E-cadherin in 21 diffuse-type tumors. The apparent differences in immunohistochemical reactivity might have resulted from destabilization of the transcript or protein.[13] Furthermore, E-cadherin immunohistochemical reactivity does not provide information about functional or structural alterations of the gene.

In the present study, we investigated the E-cadherin gene and transcript for structural alterations, as well as the status of two parental alleles by a simple and sensitive

[3] To whom requests for reprints should be addressed.

reverse transcriptase-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) method. We found that the E-cadherin gene frequently undergoes apparent two-hit inactivation in undifferentiated carcinomas of the scattered type.

MATERIALS AND METHODS

Tissue samples Twenty-four primary gastric carcinomas and corresponding normal tissues were obtained surgically from 24 patients. The tumors included 12 differentiated and 12 undifferentiated carcinomas (9 scattered-type tumors and 3 adherent-type tumors) (Table I and Fig. 1). A portion of each tissue was frozen and stored at −80°C for isolation of RNA and DNA. The remaining tissue was fixed in 10% buffered formalin for histologic examination.

Gastric carcinoma cell lines Four gastric carcinoma cell lines of the undifferentiated type (MKN-45, GCIY, HGC-27 and GT3TKB) were obtained from the Riken Gene Bank (Tsukuba Science City) and cultured under appropriate conditions (Table II).

Isolation of RNA and DNA Total RNA and DNA were isolated from 24 primary gastric carcinomas and corresponding normal tissues, as well as from four cell lines with the "TRIZOL" reagent (Gibco BRL, Life Technologies, Gaithersburg, MD).

PCR-SSCP The primers used for PCR-SSCP analysis were designed to include exon-intron boundaries where mutations have previously been reported (Table III).[14, 15]

Table I. Characteristics of Primary Gastric Carcinomas

Patient No.	Histological type	Depth of invasion	Lymph node metastasis
1	U-S[a)]	se[b)]	(+)[c)]
2	U-S	se	(+)
3	U-S	se	(+)
4	U-S (sig.)	ss	(+)
5	D	m	(−)
6	U-S (sig.)	mp	(−)
7	D	ss	(−)
8	D	ss	(+)
9	D	ss	(+)
10	D	se	(+)
11	D	se	(+)
12	U-A	ss	(−)
13	D	mp	(−)
14	U-S	se	(−)
15	U-S	se	(+)
16	D	ss	(+)
17	D	mp	(−)
18	U-S	se	(+)
19	U-A	se	(+)
20	U-A (sig.)	sm	(−)
21	D	m	(−)
22	D	sm	(−)
23	D	se	(+)
24	U-S	se	(+)

a) D, differentiated carcinoma; U-S, undifferentiated carcinoma-scattered type; U-A, undifferentiated carcinoma-adherent type; sig., signet-ring cell carcinoma.
b) m, intramucosa; sm, submucosa; mp, muscularis propria; ss, subserosa; se, exposed at serosa.
c) (+), present; (−), absent.

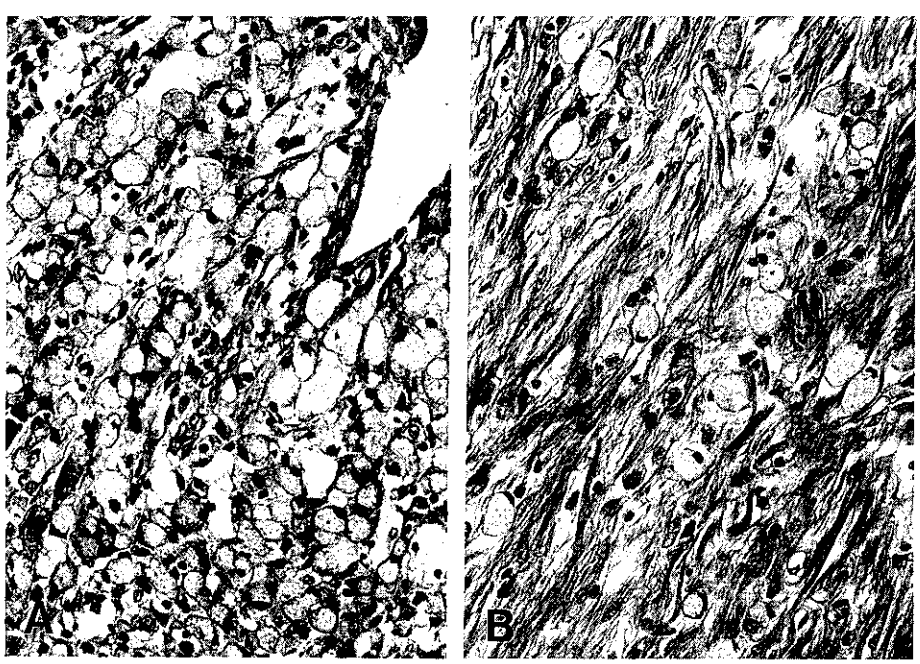

Fig. 1. Histologic features of signet-ring cell carcinomas of the adherent type (A, patient no. 20) and the scattered type (B, patient no. 4). A, Intramucosal signet-ring carcinoma cells retain cell-to-cell adhesion (hematoxylin and eosin, ×400). B, Individual signet-ring carcinoma cells infiltrate into the proliferating stroma (hematoxylin and eosin, ×400).

Extracted DNA (100 ng) was amplified in 10 μl of buffer (50 m*M* KCl, 0.01% gelatin and 10 m*M* Tris at pH 8.3) containing 10 p*M* of each primer, 1 m*M* $MgCl_2$, 0.2 m*M* of each deoxynucleotide triphosphate, 0.5 μl of [α-^{32}P] dCTP, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus Co., Norwalk, CT). PCR was performed with 40 cycles in a thermal cycler (Perkin-Elmer Cetus Co.) consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A volume of 3 μl of the PCR product was diluted 10-fold with gel-loading buffer [98% deionized formamide, 10 mmol EDTA (pH 8.0), 0.025% bromophenol blue, and 0.025% xylene cyanol], heated at 94°C for 2 min, and stored on ice until analysis. Electrophoresis was performed on a 6% neutral polyacrylamide gel with 10% glycerol at 40 W for 4 h at 4°C. The gel was fixed to Seq gel filter paper (Bio-Rad, Hercules, CA), dried on a vacuum slab gel dryer, and exposed to X-ray film at −80°C for 12–24 h.

RT-PCR and RT-PCR-SSCP Isolated RNA was reverse-transcribed using an RNA PCR kit (Perkin-Elmer Cetus Co.). The primers were designed by Becker *et al.* to amplify exon 8–9 to detect exon skipping[15] and to amplify exon 11–12 (containing an intragenic polymorphism at codon 692) to detect LOH (Table III).[16] PCR was performed under the same conditions as described for PCR-SSCP, except that radioisotope was not added in the PCR for exon 8–9. RT-PCR products (exon 8–9) were electrophoresed on 3% "NuSieve" agarose gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. SSCP analysis (exon 11–12) used the same gel as described above (40 W for 6 h at room temperature) and visualization by autoradiography.

Sequencing analysis The shifted bands detected by PCR-SSCP or RT-PCR were separated from the other PCR products and subjected to PCR using the same primers as those used in the prior PCR or internal primers by a method described previously.[17] The PCR products were purified and sequenced using a terminator cycle sequencing kit (Taq DyeDeoxy; Applied Biosystems, Foster City, CA) and a DNA sequencer (type 373A, Applied Biosystems) with the same primers as those used in the PCR or internal primers (Table III).

Table II. Characteristics of Gastric Carcinoma Cell Lines

Cell line	Characteristics	Tissue of origin
MKN-45	Poorly differentiated adenocarcinoma	Liver metastasis
GCIY	Poorly differentiated adenocarcinoma (scirrhous)	Ascites
HGC-27	Undifferentiated mucin-producing	Lymph node metastasis
GT3TKB	Undifferentiated mucin-producing (scirrhous)	Ascites

RESULTS

E-Cadherin gene mutations and exon skipping Mobility band shifts were detected by PCR-SSCP analysis in 2 (patients no. 14 and 24) of the 24 primary gastric carcinomas and in one (MKN-45) of the four gastric carcinoma cell lines (Fig. 2). In primary carcinomas, mobility band shifts were faint, and normal bands corresponding to the wild-type allele were present because of DNA contamination from non-tumorous cells. In the MKN-45 cell line normal bands were not seen, suggesting that the E-cadherin gene was inactivated completely by the mutation and concomitant loss of the wild-type allele. RT-PCR of exon 8–9 revealed an abnormal small fragment with a faint normal band in patients no. 4 and 6 (Fig.3). In patient no. 6, a larger fragment was also observed, suggesting the presence of another mRNA clone (Fig. 3), though it was not sequenced. There was no E-cadherin expression in the HGC-27 and GT3TKB cell lines (Fig. 3). In the GCIY cell line, a faint normal band may suggest a reduced expression of the E-cadherin gene (Fig. 3). Sequencing analysis revealed an A insertion at

Table III. Primer Sequences

Priming site	Upstream	Downstream
PCR-SSCP		
Exon 6	TCCTCATCAGAGCTCAAGTC	GGGTCCAAAGAACCTAAGAG
Exon 7	TGCCCAGTCCCAAAGTGCAG	TCCACACCCTCTGGATCCTC
Exon 8	AGGTGGCTAGTGTTCCTGG	CCTTTCTTTGGAAACCCTCTAA
Exon 9	GACACATCTCTTTGCTCTGC	GGACAAGGGTATGAACAGCT
RT-PCR		
Exon 8–9	ACCTCTGTGATGGAGGTC	CCACATTCGTCACTGCTACG
β-Actin	CTACAATGAGCTGCGTGTGG	ATAGCAACGTACATGGCTGG
RT-PCR-SSCP		
Exon 11–12	GTGTCCGAGGACTTTGGCGTG	TCAGAATTAGCAAAGCAAGAATTCC

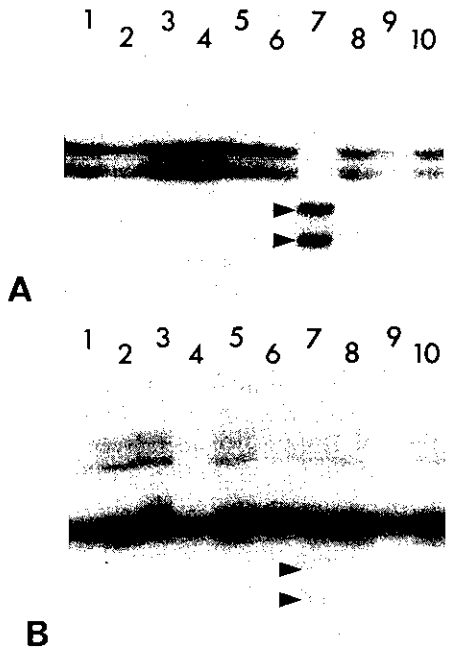

Fig. 2. PCR-SSCP analysis of exon 6 (A) and exon 9 (B) of the E-cadherin gene. A, Lanes 1–6, patients no. 19–24; lane 7, MKN-45 cell line; lane 8, GCIY cell line; lane 9, HGC-27 cell line; lane 10, GT3TKB cell line. B, Lanes 1–10, patients no. 8–17. Mobility shifts are indicated by arrowheads.

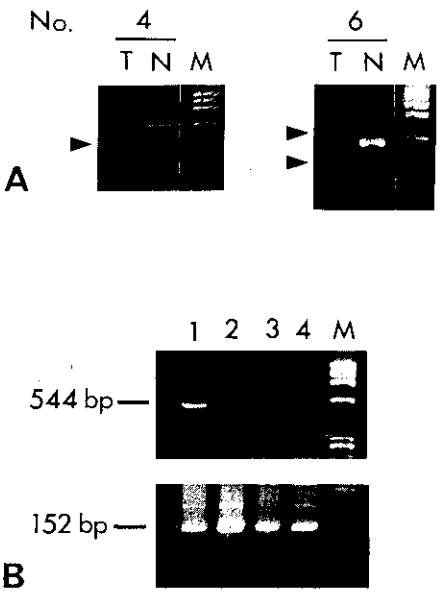

Fig. 3. RT-PCR of exon 8–9 of the E-cadherin gene in primary gastric carcinomas (A) and gastric carcinoma cell lines (B). A, Abnormal fragments in patients no. 4 and 6 are indicated by arrowheads. T, tumor RNA; N, corresponding normal RNA; M, size marker. B, Lane 1, MKN-45; lane 2, GCIY; lane 3, HGC-27; lane 4, GT3TKB; M, size marker. Exon 8–9 of the E-cadherin gene (544 bp) is not expressed in Lanes 3 and 4. β-Actin (152 bp) serves as a control.

codon 337, resulting in frame shift and skipping of exon 9 in patient no. 4 (Fig. 4), a G deletion at codon 336 (splice donor site), resulting in the skipping of exon 8 in patient no. 6, an 18 bp deletion (codon 418–423) in patient no. 14 (Fig. 4), a 3 bp deletion (codon 400, calcium-binding domain) in patient no. 24 (Fig. 4), and an 18 bp deletion at the exon 6-intron 6 boundary in MKN-45. Because all the demonstrated mutations are located at the internally repeated domains, which form a highly conserved region in the extracellular part of E-cadherin,[18] they are expected to lead to the loss of E-cadherin function. The same mutation has been detected in the MKN-45 cell line by Oda *et al.*[14] These results are summarized in Table IV.

Loss of heterozygosity (LOH) at the intragenic polymorphic site At an intragenic polymorphic site of the E-cadherin gene (codon 692), 50% (12/24) of the 24 primary carcinomas were informative for LOH. Of the 12 informative cases, LOH was identified in 4–60% (3/5) of undifferentiated (Fig. 5) and 14% (1/7) of differentiated types (Table IV). Three of the carcinomas exhibiting LOH were histologically undifferentiated carcinomas of the scattered type and demonstrated mutations or exon skipping in the remaining allele (patients no. 4, 6 and 24). No structural alteration was detected in the differentiated carcinoma with LOH (patient no. 11).

DISCUSSION

We have demonstrated that the E-cadherin gene is frequently inactivated. This inactivation occurs specifically in undifferentiated carcinomas of the scattered type via the classic two-hit mechanism, concordant with LOH and mutations in the remaining allele. Mutations and exon skipping were analyzed by PCR-SSCP, including exon-intron boundaries and RT-PCR. This was performed because mutations in exon-intron boundaries resulting in a skipping of exon 8 or 9 have previously been demonstrated in diffuse-type gastric carcinomas.[15] LOH was analyzed by RT-PCR-SSCP at an intragenic polymorphic site (codon 692).[16] This method is advantageous for detection of LOH because contamination from stromal and inflammatory cells, a particular problem for undifferentiated carcinomas of the scattered type, is diminished.[16] In addition, two parental alleles can be easily and rapidly separated by the application of SSCP.[19] Our observations are consistent with those documented in a report by Becker *et al.*,[15] in which half (13/26) of the

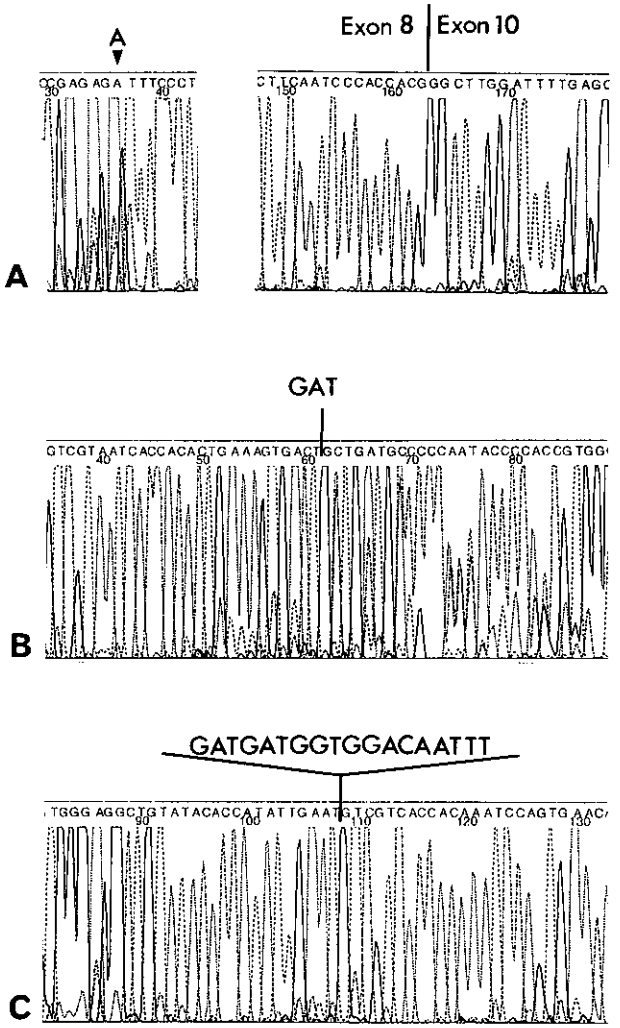

Fig. 4. Sequencing histograms of the E-cadherin gene in primary gastric carcinomas from patients no. 4 (A), 24 (B) and 14 (C). A, An insertion at codon 337 resulting in the skipping of exon 9. B, Three-bp deletion at codon 400 (calcium-binding domain). C, Eighteen-bp deletion at codon 418–423.

diffuse-type gastric carcinomas exhibited E-cadherin mutations or skipped exons. They speculated that the E-cadherin gene is a tumor/metastasis suppressor gene inactivated by LOH with mutation of the retained allele. They confirmed this two-hit inactivation in one case and only the mutated, but not normal gene transcripts, were seen in four other diffuse-type gastric carcinomas.[15] Our present results strongly support their hypothesis, because all the carcinomas carrying mutations or skipped exons exhibited simultaneous LOH at an intragenic polymorphic site of the E-cadherin gene. Becker *et al.*[16] have investigated LOH at the intragenic polymorphic site by RT-PCR followed by sequencing analysis and found identical frequencies of LOH (42%) in diffuse (8/19) and intestinal (5/12) type carcinomas. However, in our study, LOH was detected in 60% (3/5) of undifferentiated carcinomas of the scattered type and 14% (1/7) of differentiated carcinomas. In a differentiated carcinoma exhibiting LOH (patient no. 11), no structural alterations were found in the remaining allele.

As in primary gastric carcinomas, the E-cadherin gene was inactivated by the two-hit mechanism in the MKN-

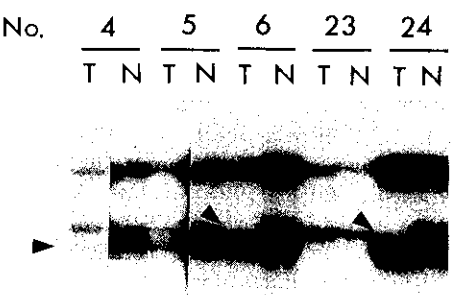

Fig. 5. RT-PCR-SSCP analysis of exon 11–12 of the E-cadherin gene. LOH is obvious in patients no. 4, 6 and 24. Patient no. 5 retains heterozygosity, and patient no. 23 is homozygous at this site.

Table IV. E-cadherin Gene Abnormalities

	Mutation	Allele status	Effect
Patient No.			
4	A insertion	LOH	skipping exon 9
6	G deletion	LOH	skipping exon 8
11	ND[a)]	LOH	N.D.
14	18 bp deletion	Hom	codon 418–423 deletion
24	3 bp deletion	LOH	codon 400 deletion (calcium-binding domain)
Cell line			
MKN-45	18 bp deletion	LOH	codon 274–277 deletion
HGC-27	ND	ND	No expression
GT3TKB	ND	ND	No expression

a) ND, not determined; LOH, loss of heterozygosity; Hom, homozygosity.

45 cell line, while the other two cell lines (HGC-27 and GT3TKB) did not express E-cadherin mRNA. The mechanisms of invasion and metastasis mediated by the inactivation of a cell adhesion system are likely to be complex. E-Cadherin-associated proteins (α-, β- and γ-catenins) have been shown to regulate E-cadherin function.[20, 21] In addition, cell adhesion molecules form complexes with the tumor suppressor APC gene and the oncogene c-*erb*B-2 or epidermal growth factor receptor.[22–24] Recently, Candidus *et al.*[25] have reported the absence of mutations of the α- and β-catenin genes regardless of the E-cadherin gene mutation status, and have suggested that alterations in the genes that regulate the expression of E-cadherin and the catenins may be the crucial factor. Yoshiura *et al.*[26] have reported that the E-cadherin gene was silenced by hypermethylation of the promoter region in several carcinoma cell lines, including gastric carcinoma lines. It is possible that the E-cadherin gene is inactivated by the hypermethylation mechanism in the HGC-27 and GT3TKB cell lines. Furthermore, CpG methylation of the E-cadherin promoter may also occur *in vivo* because gene inactivation, probably due to CpG methylation, also has been reported in primary carcinomas.[27–29] Therefore, the E-cadherin gene might be inactivated by hypermethylation in some of the undifferentiated carcinomas of the scattered type in which no structural alterations were detected in the present study.

In conclusion, the E-cadherin gene is frequently inactivated in undifferentiated carcinomas of the scattered type through the conventional two-hit mechanism. In addition, the E-cadherin gene may be inactivated by other mechanisms *in vitro* and *in vivo*, such as hypermethylation.

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